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13. ABSTRACT ***(Maximum 200 Words)***

Her-2/*neu* has been selected as a target for immunotherapy based on its prevalence on a variety of tumor cells and the poor prognosis associated with its overexpression. From a panel of a hundred α-Her-2 MAbs we have characterized eleven with the highest affinity for Her-2 for further study in the treatment of prostate cancer. Three were eventually chosen for testing as homodimers and all induced substantially more growth arrest and death *in vitro* than their corresponding monomers. Work has also begun converting a number of these MAbs into mouse/human chimeric IgG1/κ into which we have incorporated cysteine residues for homodimer formation. These genetic dimers will be evaluated for efficacy in our *in vitro* models.

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INTRODUCTION

Her-2 is the signaling transduction subunit of the Neu differentiation factor receptor (NDFR) which is comprised of Her-2 and either Her-3 or Her-4, two additional members of the EGFR family which are capable of binding NDF but are not capable of signaling (1). In carcinomas of the breast, ovary, colon and prostate, Her-2 and other components of the NDFR signaling cascade can be highly overexpressed, leading to dysregulated cell proliferation (2,3) and increased metastasis (4,5). Indeed, patients with Her-2 overexpressing tumors have the poorest prognosis (4,6,7). Antibody-mediated immunotherapy is emerging as a powerful tool to augment the effects of conventional therapy (8-10). Indeed, the first MAb for cancer therapy was approved by the FDA in July 1997. Until recently, it was assumed that most antibodies worked by virtue of their effector function, *i.e.*, ability to fix complement or recruit cytoplasmic effector cells (11-14). Based on the work which we began several years ago on lymphoma and have since extended to breast and prostate cancer, it is becoming clear that the most effective MAbs usually signal growth arrest or apoptosis in tumor cells (15-17). Importantly, negative signaling is related to hypercrosslinking, since in our studies with human lymphoma and breast carcinoma, we have discovered that MAbs which induce greater crosslinking have more inhibitory activity (15,17). This has changed the way we and others screen MAbs for clinical use. As a result, we have generated homodimers of our MAbs and have found that their anti-proliferative activity on their specific target cells *in vitro* is profoundly increased (18). Furthermore, some nonsignaling MAbs can be converted to signaling MAbs following homodimerization . This is a novel and important finding for the MAb field which has significant clinical implications. The aim of this proposal was to determine whether IgG homodimers, or $F(ab')_2$ fragments of homodimers, of our highest affinity α-Her 2 monoclonal antibodies (MAbs) alone, or in combination, would make potent anti-tumor agents in SCID mice engrafted with Her-2$^+$ human prostate cancer cell lines.

BODY:

Task 1: To prepare and test homodimers *in vitro* (grow up, purify, prepare homodimers and $F(ab')_2$ monomers and homodimers, determine K_A, K_D, structure and signaling on three cell lines.

Results: The affinity of our panel of α-HER-2 MAbs were determined by Scatchard analysis using BT474 cells, and the eleven with the best affinity, with Kds in the range of 10^{-8} to 10^{-10} M, are shown in Table 1. This panel of MAbs were shown to recognize eight non-crossblocking epitopes of HER-2.

Table 1. Characteristics of the Selected Panel of anti-HER-2 MAbs

Non-cross blocking epitope recognized on the HER-2 molecule[1]	Anti-HER-2 MAb designation[2]	Affinity ($X 10^9 M$)[1]
A	50	0.2
A	158	6.7
B	66	0.1
C	70	2
D	80	1
E	81	0.1
E	112	3.3
E	143	10
F	156	6.7
G	164	0.1
H	157	3.3

[1]Group designations randomly assigned as A-H.
[2]All are IgG1s, except HER156 which is an IgG2A.
[3]As determined by Scatchard analysis using BT474 cells.

Task 2: To characterize SCID/Xenograft model with respect to the best tumor to use, the route of administration, the growth pattern, metastases, PCR detection, endpoints etc.

Results: No progress has been made in this aim.

Task 3: To carry out all single and "cocktail" testing of homodimers and monomers on the prostate cell lines *in vitro*.

Results: Experimental results using three different α-Her-2 monomers and their respective dimers clearly showed that, in all cases, the dimers were much more effective inhibitors of proliferation at the concentrations tested (Figure 1), with IC_{50}s of 20 μg/mL for either Her50 or HER66 dimers and 40 μg/mL for HER70 dimer.

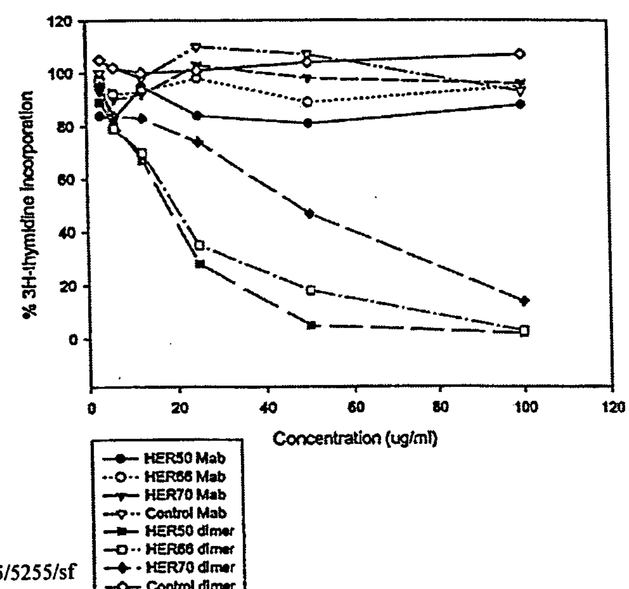

Figure 1. The effect of different MAbs monomers or dimers on [^{3}H]thymidine incorporation in LNCap cells, as described (19). Briefly, $5x10^4$ cells/well were treated for 24 hours with varying concentrations of antibody then pulsed with 1 μCi [^{3}H]thymidine for 4 hours. Wells were harvested and the radioactivity retained in the cells counted and compared with untreated cell uptake (100%).

Task 4: To determine the biodistribution and pharmacokinetics of our best monomers/dimers of IgG and F(ab')$_2$ in SCID and SCID/Xenografted mice

Results: One of the HER-2 monomers, HER66, was injected into SCID mice and its biodistribution evaluated. As shown in Figure 2, there is considerable accumulation of material in the subcutaneous tumors. The testing of the other two anti-HER-2 antibodies is in progress.

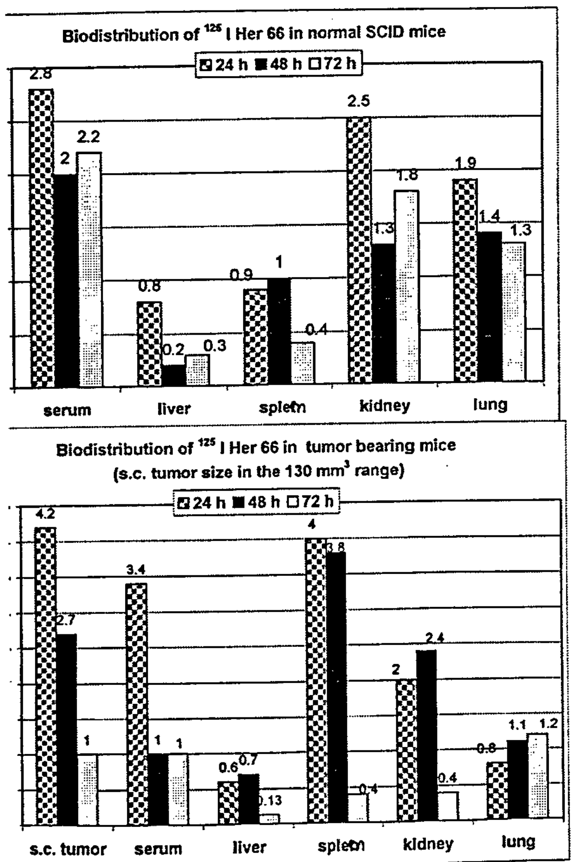

Figure 2. Biodistribution of ^{125}I HER66 in A) normal SCID mice, and B) SCID mice with 130 mm3 subcutaneous tumors. Radiolabeled α-Her2 MAb (HER66) was injected i.p. into 2 groups of 3 mice. Mice were sacrificed at 24, 48, and 72 hours. Perfused organs were weighed. The radioactivity of each organ as well as tumor and blood were measured. Results are expressed as % of injected dose per gram of tissue (%ID/g).

Task 5: To set up *in vivo* therapy experiments. The precise dose regimens will be selected based on biodistribution and pharmacokinetics

Results: Tasks 4 and 5 could not be completed being dependent on Task 2.

Task 6: cDNA cloning of the best hybridomas

Results: The variable domains of the heavy and light chains of six of the candidate anti-HER-2 antibodies (50, 66, 80, 81, 157, and 164) were PCR-amplified using a small set of primers specific for the leader sequence and the constant domain (these primers were chosen to eliminate the possibility that slightly non-homologous variable domain PCR primers would alter the sequence). Only nine of these amplifications were successful, 50H, 50L, 66H, 66L, 80L, 81H, 157H, 157L, 164L, and of these, only four were cloned and sequenced to date, 50H, 50L, 66H, and 66L.

Task 7: Express appropriate multivalent Fv constructs of cDNAs in *E coli.* Check physicochemical properties of recombinant proteins.

Results: One of these constructs, which has an extra cysteine residue incorporated near the C-terminus of the heavy chain for disulfide bond formation of an antibody homodimer, has been successfully expressed in quantities sufficient to be converted into dimers using treatment with Elman's reagent. Characterization of this dimer, including avidity, specificity, and signaling properties will be evaluated and compared to the monomeric chimeric form. Two other chimeric constructs were described, one in which a cysteine residue was placed at the penultimate position of a 17-mer peptide at the C-terminal end of the heavy chain to facilitate homodimer formation, the other with an scFv of the same specificity genetically grafted onto the C-terminal of the heavy chain to generate a tetravalent construct. Both of these constructs have been successfully engineered, however, the yields from non-secreting mouse myeloma cell lines transfected by electroporation were never adequate for any characterization. Alternate transfection methods are being investigated that have since proven superior in our lab, including lipofection.

KEY RESEARCH ACCOMPLISHMENTS:

- The panel of anti-HER-2 MAbs was evaluated for binding affinity to HER-2 and the best eleven chosen for further evaluation.

- Three of the best anti-HER-2 MAbs, HER50, Her66 and HER70, were chemically dimerized and were shown to be significantly more cytotoxic than their corresponding monomers.

- A biodistribution study using HER66 showed that this antibody does accumulate in a tumor.

- Variable domain cloning of the light and heavy chain of six of the a-HER-2 MAbs has been initiated; two, HER50 and HER66 clones have been sequenced and await insertion into the chimeric, dimer expression vectors.

- Three different genetic dimer/multimer genetic constructs have been made and all express in transfected myeloma cells, though two of these in quantities that are not sufficient for evaluation.

REPORTABLE OUTCOMES: None

CONCLUSIONS:

We have identified three anti-HER-2 homodimers with antiproliferative activity on LnCAP cells *in vitro*. Because the generation of chemical homodimers is expensive, gives low yields and several biproducts, we are in the process of generating recombinant homodimers using three different strategies. One of these constructs has expression levels high enough to produce material for further evaluation. Six anti-HER-2 MAbs light and heavy chain variable domain regions are in various stages of cloning; these will be inserted into the best recombinant homodimer construct for expression. These genetic homodimers will be evaluated *in vitro* and *in vivo* using a SCID mouse xenograft model.

REFERENCES:

1. Karunagaran, D., E. Tzahar, R.R. Beerli, X. Chen, D. Graus-Porta, B.J. Ratzkin, R. Seger, N.E. Hynes, and Y. Yarden. 1996. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: Implication for breast cancer. EMBO J. **15**: 254-264.

2. Bacus, S.S., C.R. Zelnick, G. Plowman, and Y. Yarden. 1994. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. Am.J.Clin.Pathol. **102**: S13-S24

3. Di Fiore, P.P. and Kraus, M.H. Mechanisms involving an expanding erbB/EGF receptor family of tyrosine kinases in human neoplasia. In: *Genes, oncogenes, and hormones: Advances in cellula and molecular biology of breast cancer*, edited by Dickson, R.B. and Lippman, M.E. Boston: Kluwer Academic Publishers, 1991,

4. Giai, M., R. Roagna, R. Ponzone, M. De Bortoli, C. Dati, and P. Sismondi. 1994. Prognostic and predictive relevance of c- *erbB*-2 and *ras* expression in node positive and negative breast cancer. Anticancer Res. **14**: 1441-1450.

5. Tan, M., J. Yao, and D. Yu. 1997. Overexpression of the c-*erb*B-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. Cancer Res. **57**: 1199-1205.

6. Hoskins, K. and B.L. Weber. 1994. The biology of breast cancer. Curr.Opin.Oncol. **6**: 554-559.

7. Grizzle, W.E., R.B. Myers, M.M. Arnold, and S. Srivastava. 1994. Evaluation of biomarkers in breast and prostate cancer. J.Cell.Biochem. **Supplement 19**: 259-266.

8. Maloney, D.G., Levy, R., and Campbell, M.J. Monoclonal antibody therapy. In: *The molecular basis of cancer*, edited by Mendelsohn, J. Philadelphia: W.B. Saunders Company, 1995, p. 460-510.

9. Goldenberg, D.M. 1993. Monoclonal antibodies in cancer detection and therapy. Am.J.Med. **94**: 297-312.

10. Pietersz, G.A., Krauer, K., and McKenzie, I.F.C. The use of monoclonal antibody immunoconjugates in cancer therapy. In: *Antigen and antibody molecular engineering in breast cancer diagnosis and treatment*, edited by Ceriani, R.L. New York: Plenum Press, 1994, p. 169-179.

11. Hooijberg, E., P.C.M. van den Berk, J.J. Sein, J. Wijdenes, A.A.M. Hart, R.W. de Boer, C.J.M. Melief, and A. Hekman. 1995. Enhanced antitumor effects of CD20 over CD19 monoclonal antibodies in a nude mouse xenograft model. Cancer Res. **55**: 840-846.

12. Hooijberg, E., J.J. Sein, P.C.M. van den Berk, A.A.M. Hart, M.A. van der Valk, W.M. Kast, C.J.M. Melief, and A. Hekman. 1995. Eradication of large human B cell tumors in nude mice with unconjugated CD20 monoclonal antibodies and interleukin 2. Cancer Res. **55**: 2627-2634.

13. Riethmuller, G., E. Schneider-Gadicke, and G. Schlimok. 1994. Randomized trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. Lancet **343**: 1177-1183.

14. Dyer, M.J.S., G. Hale, F.G.J. Hayhoe, and H. Waldmann. 1989. Effects of CAMPATH-1 antibodies in vivo in patients with lymphoid malignancies: influence of antibody isotype. Blood **73**: 1431-1439.

15. Marches, R., E. Racila, T.F. Tucker, L. Picker, P. Mongini, R. Hsueh, E.S. Vitetta, R.H. Scheuermann, and J.W. Uhr. 1996. Tumor dormancy and cell signalling III: Role of hypercrosslinking of IgM and CD40 on the induction of cell cycle arrest and apoptosis in B lymphoma cells. Therap.Immunol. **2**: 125-136.

16. Ghetie, M.A., L.J. Picker, J.A. Richardson, K. Tucker, J.W. Uhr, and E.S. Vitetta. 1994. Anti-CD19 inhibits the growth of human B-cell tumor lines *in vitro* and of Daudi cells in SCID mice by inducing cell cycle arrest. Blood **83**: 1329-1336.

17. Racila, E., R.H. Scheuermann, L.U. Picker, E. Yefenof, T. Tucker, W. Chang, R. Marches, N.E. Street, E.S. Vitetta, and J.W. Uhr. 1995. Tumor dormancy and cell signaling II. Antibody as an agonist in inducing dormancy of a B cell lymphoma in SCID mice. J.Exp.Med. **181**: 1539-1550.

18. Ghetie, M.-A., E.M. Podar, A. Ilgen, B.E. Gordon, J.W. Uhr, and E.S. Vitetta. 1997. Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells. Proc.Natl.Acad.Sci.USA **94**: 7509-7514.

19. Ghetie, M.-A., R.D. May, M. Till, J.W. Uhr, V. Ghetie, P.P. Knowles, M. Relf, A. Brown, P.M. Wallace, G. Janossy, P. Amlot, E.S. Vitetta, and P.E. Thorpe. 1988. Evaluation of ricin A chain-containing immunotoxins directed against CD19 and CD22 antigens on normal and malignant human B- cells as potential reagents for in vivo therapy. Cancer Res. **48**: 2610-2617.

APPENDICES: None